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Evaluation of proliferation parameters in in vivo bromodeoxyuridine labelled lung cancers

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Abstract In a series of 44 bronchial biopsies from patients suspected of having endobronchial lung carcinoma, the validity of proliferating cell nuclear antigen (PCNA) and Ki67 antigen as proliferative indicators was evaluated in ethanol fixed, paraffin embedded tissue. The percentages of cells positive for these markers were compared to the in vivo bromodeoxyuridine (BrdU) labelling index. A good correlation was found between PCNA immunoreactivity and BrdU labelling index, while Ki67-antigen expression showed a significant relation with BrdU labelling index and with PCNA expression. All three parameters showed a trend towards similar values for the individual cases. Based on the fact that Ki67 antigen is expressed in all cycling cells, whereas replicon-associated PCNA and BrdU only reflect the S-phase fraction, the differences between Ki67-antigen scores on the one hand and BrdU and PCNA scores on the other were smaller than expected. In order to determine the degree of concordance between immunohistochemically and flow cytometrically detected proliferation variables, BrdU incorporation was measured using both methods in duplicate bronchial specimens. Discrepancies in labelling indices were observed predominantly in DNA diploid samples, with consistently lower values in the flow cytometrically analysed specimens. In tumour specimens with an aneuploid DNA content, flow cytometric determination of proliferative activity yielded results similar to those obtained by tissue section examination. We conclude that the scores for PCNA and Ki67 antigen, immunohistochemically detected in ethanol fixed, paraffin embedded tissue reflect functional proliferative activity.

Key words Bromodeoxyuridine · Flow cytometry · Immunohistochemistry · Ki67 antigen · Proliferating cell nuclear antigen

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Introduction

Immunohistochemical studies of proliferative activity show that the detection of cell cycle parameters in tumour tissue sections may be a useful tool in the determination of prognostic indicators and in the evaluation of treatment response [10, 11, 17, 39, 46, 53]. The use of immunohistochemical methods to detect molecules involved in cell cycle regulation and DNA replication is nowadays instrumental in this approach, in particular since it has became applicable to paraffin embedded tumour material.

Two of the most commonly used proliferation-associated proteins are proliferating cell nuclear antigen (PCNA) and Ki67 antigen. PCNA functions as an auxillary protein for polymerase-δ [4, 33, 42] and thus appears to be involved in DNA replication [32, 41]. The synthesis of PCNA reaches its maximum during the S-phase and is rapidly degraded thereafter [7, 26]. In this study we have used a monoclonal antibody (PC10) that recognizes PCNA in routinely fixed and histologically processed material developed by Waseem and Lane [52].

The Ki67 antigen comprises a nuclear matrix protein expressed in all cycling cells [15, 50, 51] and in the past years, Ki67-antigen staining has become a standard procedure in the assessment of the extent of cell proliferation [5]. Recently, monoclonal antibodies have been developed that are directed against Ki67-antigen epitopes

resistant to formalin fixation [22], MIB-1 giving the best results after microwave pretreatment [6].

When comparing reports in the literature on the expression of PCNA and Ki67 antigen, one is often confronted with inconsistent results. Fixation and staining methods appear to be crucial in influencing the final scores [12, 27]. For example, at least two intracellular forms of PCNA exist [3], a replicon-associated form, which is resistant to alcohol fixations and non-ionic detergents, and an alcohol soluble form [8]. The latter is not associated with S-phase activity and is present in almost all cycling cells. Therefore, alcohol fixation or extraction is applied to cells and tissues to be subjected to cell cycle dependent PCNA staining.

The immunohistochemical assessment of PCNA and Ki67 antigen in tissue sections may pose some difficulties with respect to the interpretation of the data. First of all, it is often not evident to what extent the data obtained by immunohistochemistry represent actual proliferation. For example, Ki67 antigen is only weakly expressed in early G_1 cells [2, 15], and the procedure may therefore fail to detect these cells. Several authors have reported relatively low Ki67 antigen scores as compared to other proliferation indicators, such as PCNA or bromodeoxyuridine (BrdU) [19, 21, 54]. Furthermore, cells in culture show loss of Ki67 antigen immunoreactivity during nutritional deprivation [1, 48, 51].

In this study, the expression of PCNA and Ki67 antigen is compared to in vivo BrdU incorporation in ethanol fixed, paraffin embedded bronchial biopsies. Our aim was to determine the validity of these markers as quantitative indicators of proliferation. BrdU, a thymidine analogue, is incorporated into the newly replicated DNA of S-phase cells. Therefore, the BrdU labelling index is a measure of functional proliferative activity [31, 47]. An established method to detect the BrdU labelling index is flow cytometry and although this technique has the advantage of analysing a large number of cells when compared with immunohistochemical evaluation of tissue sections, the advantage of the latter method is that the tissue architecture can be included in the evaluation and individual proliferating cells can be visualized in their local context. Flow cytometric analysis may also be disturbed by the admixture of non-malignant cells, such as stromal cells. Since this problem is particularly prominent in biopsy samples, which are usually small sized, we have determined the correlation between immunohistochemically and flow cytometrically detectable BrdU incorporation in such specimens.

Materials and methods

The bronchoscopic biopsies used in this study were duplicate specimens of the biopsies obtained for the study described by Tinnemans et al. [43]. Approval for in vivo labelling with BrdU was given by the ethical committee of the University Hospital Maastricht. After informed consent, the patients were infused with 50 mg/m^2 BrdU (Janssen Pharmaceutica, Beerse, Belgium), dissolved in 100 ml 0.9% sodium chloride, within a timespan of 10 min. After approximately 4–5 h, biopsies were taken with a flexible bronchoscope, fixed in formalin for routine diagnosis and in cold (4° C) 70% ethanol for the immunohistochemical studies. The latter samples were stored at 4° C until use. Specimens from 38 males and 6 females were included in this study. The evaluated cases were divided into two groups: a group with tumour tissue or at least carcinoma in situ (CIS) found in the biopsy (tumour group) and a group with normal bronchial epithelium or metaplasia found in the biopsy (non-tumour group). Each specimen was allocated to only one group. Almost all patients had bronchial carcinoma although this was not always detectable in the biopsies obtained for this study. In Table 1 the distribution of different types of bronchial carcinoma is given for these 44 patients,

For PCNA staining we used the monoclonal antibody PC10 [52], kindly supplied by Dr. D. Lane (Dundee, UK). Ki67 antigen was detected using the monoclonal antibody MIB-1 [22], which was a gift from Dr. J. Gerdes (Forschungsinstitut Borstel, Germany). The monoclonal antibody against BrdU (clone IIB5) was raised and characterized as described before [36].

For immunohistochemistry 44 bronchial specimens, fixed in 70% ethanol, were paraffin embedded and 4 µm sections were cut. After removal of paraffin by xylene and rehydration, endogenous peroxidase activity was blocked in methanol containing 0.3% hydrogen peroxide for 20 min. Slides were rinsed in phosphate buffered saline (PBS) and immunohistochemical procedures were applied. Different procedures were followed for immunostaining of BrdU, PCNA and Ki67 antigen.

For BrdU staining slides were incubated in 2 N hydrochloric acid (HCl) for 30 min at 37 °C to denature nuclear DNA, followed by two washing steps in borax buffer (0.1 M sodium tetraborate in destilled water, pH 8.5) and two washing steps in PBS. The slides were then incubated with appropriately diluted anti-BrdU antibody for 60 min at 37 °C. After rinsing in PBS, primary antibody binding was visualized by incubation with peroxidase conjugated rabbit anti mouse IgG (DAKO, Glostrup, Denmark, code P260) diluted 1:200 in PBS, for 60 min at room temperature (RT). 3,3´-Diaminobenzidine tetrahydrochloride (Sigma, St. Louis, Mo., USA) was used as the chromogen. Finally, the sections were lightly counterstained with haematoxylin, dehydrated and coverslipped.

PCNA staining was carried out on slides incubated with the monoclonal antibody PC10, diluted 1:25 in PBS, for 60 min at 37 °C. After a washing step in PBS, primary antibody binding was visualized with peroxidase-conjugated rabbit anti mouse IgG, and the slides were further processed as described above.

Ki67-antigen staining was performed as follows. Slides were placed in a slide rack and immersed in 500 ml 0.01M citrate buffer (pH 6.0) in a plastic microwave container. They were boiled for 15 min at maximum power (850 W) in a microwave oven (Moulinex Oscillotron). During this process, slides were kept immersed in buffer. Then, slides were allowed to cool down to RT for 60 min. They were rinsed in PBS and incubated for 60 min at RT with

Table 1 Distribution of lung cancer type and stage of patients from which the biopsies were derived (*AC* adenocarcinoma, *ED* extended disease, *LCLC* large cell lung cancer, *LD* limited disease, *I-IV* staging according to UICC criteria, *NM* non-malignant, *NT* non-tumour group, *SCLC* small cell lung cancer, *SQC* squamous cell carcinoma, *T* tumour group)

	SCLC		SQC				LCLC				AC				NM	Total
	ED	LD	I	II	III	IV	I	II	III	IV	I	II	III	IV		
NT	5		1	1	4		2		4				1	1	3	22
T	2	1			4	7			4	1			3			22

MIB-1 antibody, diluted 1:20 in PBS. After a washing step in PBS, primary antibody binding was visualized by incubation with peroxidase conjugated rabbit anti mouse IgG, and slides were further processed as described above.

Positive controls consisted of in vivo BrdU-labelled human tumour specimens known to be positive for BrdU, PCNA and Ki67 antigen. Negative controls consisted of non-BrdU labelled human colon specimens. In control incubations the primary antisera were replaced by PBS or, in case of a control for anti-BrdU, the HCl denaturation step was omitted.

All sections were evaluated under the supervision of an experienced pathologist (SjScW). Tumour tissue areas, as well as areas displaying normal tissue, metaplasia or dysplasia, were marked. In these different areas, the percentages of cells showing specific BrdU, PCNA or Ki67 antigen expression were determined. No correction was made for division of BrdU labelled cells in the time lapse between BrdU administration and biopsy sampling, since the fraction of BrdU labelled G_1 cells was found to be negligible. Three series of 200 cells were counted in various representative fields in each section in order to minimalize intraobserver variations. Stained nuclei were considered positive, irrespective of intensity. In biopsies with heterogeneously staining tumour fields, the fields examined included those with the highest and those with the lowest percentage of stained cells. The results were averaged.

Not all biopsies contained sufficient material to evaluate all three parameters. In several slides, part of the epithelial tissue was damaged or completely lost. These areas were omitted from evaluation, and so were tangentially cut areas in order to avoid under- or overestimation of the growth fraction.

Dysplastic tissue and tumour tissue were often found together within one section. These cases were included in the tumour group and only the areas containing tumour cells were analysed.

Histograms showed serious skewing of the data and unequal standard deviations were observed between the two groups. To correct for the skewing, a logarithmic transformation was performed [23]. To this end, all data were upgraded by 1, to include zero values. Transformation to a different scale also resulted in approximately equal standard deviations, thus allowing the use of a Student's *t*-test (with pooled variance estimate) for comparison of two samples.

Table 2 Averages and ranges of scores for proliferating cell nuclear antigen (*PCNA*), Ki67 antigen and bromodeoxyuridine (*BrdU*) positive cells in the non-tumour and tumour group. *P*-values were obtained using a two-sample *t*-test after logarithmic transformation of the data

	% PCNA$^+$ (range)	% Ki67$^+$ (range)	% BrdU$^+$ (range)
NT	1 (0–7)	3 (0–22)	3 (0–8)
T	24 (0–63)	24 (3–78)	17 (3–46)
P-value	2×10^{-7}	3×10^{-8}	5×10^{-7}

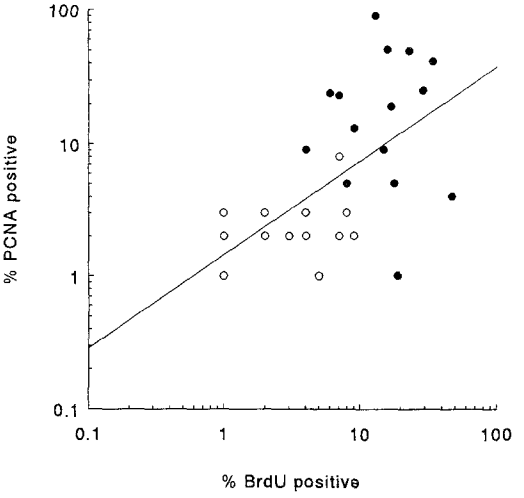

Fig. 1 Percentages of cells with bromodeoxyuridine (BrdU) labelling (*horizontal axis*) versus cells expressing proliferating cell nuclear antigen (PCNA; *vertical axis*). A significant correlation was found (P=0.00054). Data from the non-tumour group (*open circles*) as well as from the tumour group (*filled circles*) are included

Results

Fourty-four bronchial biopsies of patients suspected of having endobronchial lung carcinoma were analysed (38 males, 6 females). In 22 cases, only normal bronchial epithelium was found. In some of these biopsies dysplasia or metaplasia occurred next to normal tissue. The remaining 22 biopsies clearly showed regions of tumour.

Since some of the sections obtained from one biopsy only contained stromal tissue and could not be replaced by another section because of size, only 68% of the cases could be analysed for all three parameters. In 32% of the biopsies only one or two parameters could be evaluated.

Comparison of proliferation scores

The non-tumour and tumour groups were compared with regard to average scores of PCNA, Ki67 antigen and BrdU positivity. In general, biopies in which carcinoma cells or CIS were detected showed elevated proliferative markers when compared with biopsies in which no tumour tissue was found (Table 2). Large standard deviations were observed for each variable. In a two sample *t*-test using logarithmically transformed data, *P*-values lower than 0.05 indicated a significant difference between the proliferation parameter scores of the non-tumour and the tumour group. Comparison of average Ki67 antigen and BrdU values on the one hand, and of average Ki67 antigen and PCNA values on the other within the tumour group, revealed a lower number of Ki67 antigen-positive cells than would be expected, considering the fact that Ki67 antigen is expressed in all cycling cells whereas PCNA and BrdU only reflect the S-phase fraction.

The paired values for the proliferation markers Ki67 antigen and PCNA were compared with each other and with BrdU incorporation data (Figs. 1–3). Logarithmically transformed data were used. When individual PCNA scores were compared with the corresponding BrdU labelling indices, a significant correlation (P<0.05) was found between these markers. Similarly, a significant correlation between BrdU labelling index and Ki67 antigen expression and between PCNA and Ki67 antigen expression was seen (P<0.05). All three parameters showed a trend towards similar values for the individual cases.

In order to evaluate the degree of concordance between flow cytometry and immunohistochemistry, we

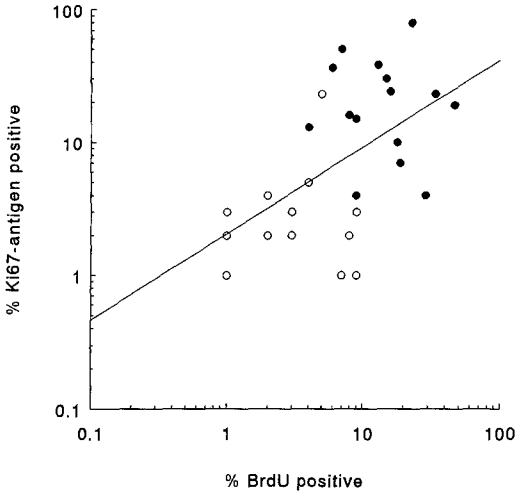

Fig. 2 Percentages of cells with BrdU labelling (*horizontal axis*) versus cells expressing Ki67 antigen (*vertical axis*). A significant correlation was found (P=0.002)

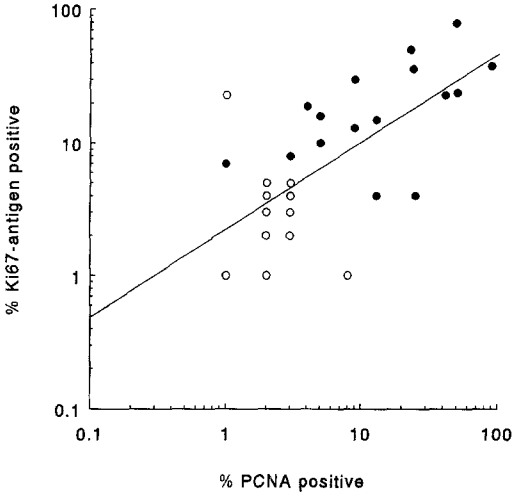

Fig. 3 Percentages of cells expressing PCNA (*horizontal axis*) versus cells expressing Ki67 antigen (*vertical axis*). A significant correlation was found (P=0.00002)

compared parallel biopsies in which the BrdU labelling index was determined either in the tissue sections or in cell suspensions in 32 cases. We expected that differences, if any, in outcome between flow cytometry and immunohistochemistry would be most prominent in diploid tumours, given the problem of admixture of non-proliferating tissue. Therefore, we distinguished between DNA diploid and DNA aneuploid biopsies. Figure 4 shows the BrdU labelling index, determined from sections of ethanol fixed, paraffin embedded cells and the BrdU labelling index of parallel biopsies obtained from the same patients, flow cytometrically determined from ethanol fixed nuclei in a former study [43]. In DNA diploid biopsies, the BrdU labelling index is underestimated using flow cytometry. In DNA aneuploid biopsies, the differences seemed more random and no systematic under- or overestimation was observed using either of the two methods.

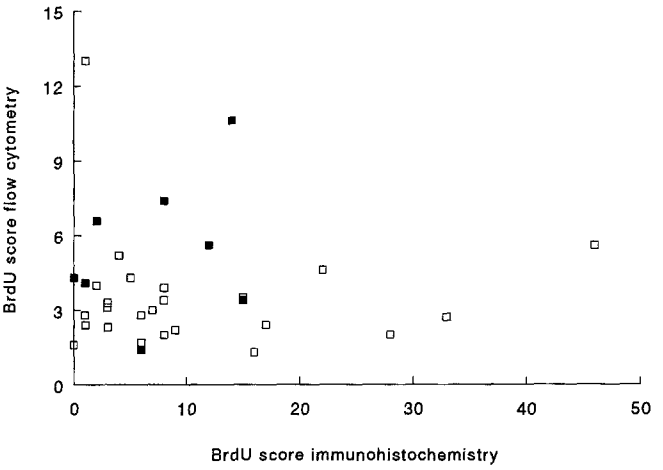

Fig. 4 BrdU labelling indices determined immunohistochemically on sections (*horizontal axis*) versus BrdU labelling indices determined flow cytometrically in suspension (*vertical axis*). Data from DNA diploid samples (*open squares*) as well as from DNA aneuploid samples (*filled squares*) are included

Discussion

The proliferation markers PCNA and Ki67 antigen were compared with the vivo BrdU incorporation in a series of bronchial biopsies, comprising 22 samples containing lung carcinomas and 22 samples with normal bronchial epithelium and/or metaplasia. In most of the comparative studies performed [20, 25, 30, 35, 49], BrdU was administered after excision of the tissue or in vitro. However, the ex vivo labelling assays are sensitive to technical variability, since they rely on an efficient diffusion of the label, while the results of cell culture assays often do not reflect the growth conditions in situ.

In concordance with the BrdU labelling indices, scores of Ki67 antigen and PCNA were significantly elevated in the tumour group when compared with the non-tumour group. The high variance in the tumour group may reflect the inter- and intra-tumoral heterogeneity in proliferation which is a well established phenomenon [40].

PCNA versus BrdU

We found a significant correlation between individual BrdU and PCNA scores. This is consistend with the assumption that the replication-associated form of PCNA remaining in the cell after alcoholic fixation, reflects the S-phase fraction [8]. In an immunocytochemical study of PCNA expression and BrdU incorporation in lung cancer cell lines, we observed that both are found in the same cell population and colocalize in the nucleus (Schutte et al., unpublished data). The same was described by Humbert et al. [20], who reported a comparable distribution of PCNA and BrdU within the nuclei of methanol-fixed MCF-7 cells. Sasaki et al. [34] also found matching dis-

tribution patterns of PCNA and BrdU in methanol fixed, Triton X-100 extracted cells. PCNA, detected in ethanol fixed paraffin embedded tissue, thus appears to be a reliable indicator of functional proliferative activity. Reports suggest that the detectability of PCNA is strongly dependent on the type and duration of fixation [3, 12, 13, 14, 24, 27, 34]. In studies where formalin was used as a fixative, PCNA scores are often reported to be higher than the corresponding BrdU labelling indices [9, 29, 38], or than the flow cytometrically determined S-phase fraction [18], probably due to the formalin resistant, non-specific form of PCNA. Studies describing the use of alcoholic fixatives usually report a good correlation between PCNA expression and BrdU labelling index [20, 25, 30, 35, 49].

Ki67 antigen versus BrdU and PCNA

A significant correlation was found when Ki67 antigen expression was compared with BrdU incorporation and with PCNA expression. Being expressed in all cycling cells [15], Ki67 antigen immunoreactivity was expected to exceed the labelling indices of PCNA and BrdU, which merely represent the S-phase fraction. However, we did not observe a systematically higher Ki67 antigen score. An explanation for this finding may be that Ki67 antigenicity is lost as a result of environmental factors. Verheijen et al. [51] reported loss of Ki67 antigen reactivity in nutritionally deprived cells which were still in S, G_2 or M-phase, as measured by flow cytometry. Similarly, Baisch and Gerdes [1] and Van Dierendonck et al. [48] reported loss of correlation between Ki67 antigen immunoreactivity and proliferation during nutrient deprivation. In a study of nutritionally depleted small cell lung cancer cells, a gradual disappearance of PCNA and Ki67 antigen was observed, showing a more rapid decline of the latter [44]. Another explanation for the low Ki67 antigen scores is that cells express Ki67 antigen below the detection level. Lack of Ki67 antigenicity in early G_1 cells is a frequently described phenomenon [2, 15, 16]. Tsurusawa and Fujimoto [45] reported a down regulation of Ki67 antigen expression to an undetectable level in tumour cells with relatively long G_1 duration. Landberg and Roos [28] found that G_1 cells in the first cell cycle are Ki67 antigen negative, while continuously cycling cells express Ki67 antigen also in G_1.

Flow cytometry versus immunohistochemistry

To investigate the concordance between flow cytometry and immunohistochemistry in the evaluation of proliferation markers, we compared BrdU labelling indices determined from immunohistochemically stained sections with data of duplicate bronchial specimens, evaluated flow cytometrically. The small discrepancies that were observed in DNA aneuploid tumours seemed random, and can be explained by differences in sample size. The DNA diploid samples however, the discrepancies observed were more extensive. A likely explanation for this phenomenon is the admixture of non-proliferative cells in the flow cytometric sample, resulting in an underestimation of the labelling index. Although, in contrast to the tissue section method, the use of flow cytometry enables the evaluation of large cell numbers, this technique does not enable selection of relevant cells. This problem is predominantly present in DNA diploid samples, where DNA ploidy cannot serve as a selection criterion. A solution for this problem is immunostaining with cytokeratin prior to or simultaneously with staining for BrdU, PCNA or Ki67 antigen. This procedure has been proven feasible by Schutte et al. [37], who showed that three parameter analysis of patient biopsies offers the possibility of enriching the tumour cell population by gating on epithelial cells.

In conclusion, our results indicate that scores for PCNA and Ki67 antigen expression are reliable indicators of proliferative activity in ethanol fixed, paraffin embedded tissue. The results obtained for these two cell cycle parameters correlate significantly with in vivo incorporated BrdU values. These findings therefore justify the diagnostic use of PCNA and Ki67 antigen.

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